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TITLE OF INVENTION

A METHOD OF TREATMENT AND AN ANIMAL MODEL USEFUL FOR SAME

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

Courtesy copy of international application
Six (6) sheets of Drawings

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A METHOD OF TREATMENT
AND AN ANIMAL MODEL USEFUL FOR SAME

5 **FIELD OF THE INVENTION**

The present invention relates generally to a method of treatment and to an animal model for the identification of molecules and genetic sequences useful in a method of treatment including inducing or reducing the fertility of male animals. More particularly, the present invention contemplates a method for the treatment of infertility or a method of reducing
10 fertility and even more particularly a method for modulating spermatogenesis in an animal or avian species. There is also provided an animal model comprising a mutation in at least one allele of *bcl-w* or in a gene associated with *bcl-w*. Such animals fail to undergo productive spermatogenesis and can be used to screen for therapeutic molecules including genetic sequences capable of inducing, enhancing or otherwise facilitating spermatogenesis in said
15 animals as well as a model for molecules and genetic sequences which can induce infertility.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

20

Considerable effort has and continues to be expended on therapeutic protocols for the treatment of genetically based disorders. To facilitate the rationale design of such therapeutic protocols, scientists first need to understand and elucidate the biochemical and genetic intricacies of intracellular pathways and physiological processes. Several key regulators have
25 been identified which have involvement in intracellular pathways and physiological processes. A particularly important group of proteins is the Bcl-2 family of proteins.

Bcl-2 is a 26 kDa cytoplasmic protein encoded by the *bcl-2* gene translocated to the IGH locus in human follicular lymphoma and is regarded as the prototypic mediator of cell survival
30 (1). The Bcl-2 proteins have a role in controlling cellular apoptosis. Apoptosis is a

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morphologically distinctive and genetically programmed process of cell death (2) and plays an important role in embryogenesis, tissue homeostasis and the immune system.

Disrupted regulation of apoptosis is strongly implicated in cancer and in autoimmune and degenerative diseases. Key regulators include proteins of the Bcl-2 family (reviewed in 3-5), some of which (eg Bcl-2, Bcl-x_L, Mcl-1 and A1) promote cell survival while others (eg Bax, Bak) act as antagonists. Because members of these opposing factions can associate and seemingly titrate one another's function, their relative abundance in a particular cell type may determine its threshold for apoptosis (6). The competitive action of the pro- and anti-survival Bcl-2-related proteins regulates the activation of the proteases (caspases) that dismantle the cell, but how they do so remains uncertain (3-5). The pro-survival proteins may, however, associate with caspase-activating adaptors such as Ced-4 and Apaf-1 and prevent their activity (7-8) and/or prevent the release of pro-apoptotic proteins from mitochondria (9, 10, 11).

The pro-survival family members are expressed in diverse tissues in distinct but overlapping patterns. While their biochemical actions are difficult to distinguish, gene inactivation studies suggest that each may have critical roles in particular tissues. Mice which lack Bcl-2 develop normally, but later display marked lymphocytopenia, polycystic kidney disease, hypopigmented hair, motoneuron degeneration and disordered growth of intestinal villi and long bones (12-17). In contrast, mice which lack Bcl-x_L die *in utero* due to massive apoptosis of both hematopoietic and neuronal cells (18).

Bcl-w is a pro-survival protein identified by the present inventors (19; International Patent Application No. PCT/AU97/00199, filed 27 March, 1997 and incorporated herein by reference). Enforced expression of *bcl-w*, like *bcl-2*, renders myeloid and lymphoid cell lines refractory to apoptosis induced by cytokine deprivation or irradiation, but is relatively ineffective against apoptosis induced by engagement of the CD95 (Fas) 'death' receptor. Transcripts of *bcl-w* are present at moderate levels in brain, colon and salivary gland, and at low levels in testis, liver, heart, stomach, skeletal muscle and placenta, as well as in most myeloid cell lines but few lymphoid lines (19).

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In work leading up to the present invention and in order to identify in which tissues Bcl-w plays an essential role, the inventors undertook *bcl-w* gene disruption studies in mice. It has now been surprisingly determined that mice deficient for *bcl-w* and/or a gene associated with *bcl-w* fail to undergo productive spermatogenesis and are infertile without showing any other major abnormality. In contrast, Bcl-w is apparently dispensable in other tissues. The mice provide, therefore, a useful model for studying infertility in animal and avian species.

SUMMARY OF THE INVENTION

Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined following the bibliography.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

One aspect of the present invention is directed to a modified animal or avian species exhibiting reduced levels of a Bcl-w protein and/or a protein associated with Bcl-w or a derivative or homologue thereof, wherein said animal or avian species has an incapacity or a reduced capacity to induce or facilitate spermatogenesis.

Another aspect of the present invention provides a modified animal or avian species exhibiting reduced levels of a Bcl-w protein having an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:4 or a Bcl-w protein encoded by a nucleotide sequence substantially set forth in SEQ ID NO:1 or SEQ ID NO:3 or a nucleotide sequence capable of hybridising to SEQ ID NO:1 or 3 or 5 or 7 under low stringency conditions at 42 °C wherein said animal or avian species has an incapacity or a reduced capacity to induce or facilitate spermatogenesis.

Yet another aspect of the present invention provides a modified animal exhibiting reduced levels of Bcl-w or a derivative or homologue thereof and/or of a protein associated with

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Bcl-w wherein said Bcl-w or its derivative or homologue comprises an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:4 or an amino acid sequence having at least about 47% similarity to the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4 and wherein said modified animal has an incapacity or a reduced capacity to induce or facilitate
5 productive spermatogenesis.

Still yet another aspect of the present invention contemplates a modified animal exhibiting reduced levels of Bcl-w or a derivative or homologue thereof and/or of a protein associated with Bcl-w wherein said Bcl-w or its derivative or homologue is encoded by a nucleotide
10 sequence substantially as set forth in SEQ ID NO:1 or SEQ ID NO:3 or a nucleotide sequence having at least 47% similarity thereto and/or which can hybridise to SEQ ID NO:1 or SEQ ID NO:3 under low stringency conditions at 42°C.

Another aspect of the present invention is directed to a modified animal exhibiting an
15 incapacity or a reduced capacity to induce or facilitate productive spermatogenesis said modification comprising the administration to said animal of an antagonistic effective amount of a molecule capable directly or indirectly of antagonising Bcl-w protein activity or the ability of a derivative or homologue of Bcl-w.

20 Yet another aspect of the present invention provides a composition capable of inducing infertility or reducing fertility in an animal, said composition comprising a direct or indirect antagonist of a Bcl-w protein.

Still yet another aspect of the present invention relates to a genetically modified animal
25 comprising a mutation in one or more alleles of a gene encoding a Bcl-w protein and/or of a gene encoding a molecule associated with Bcl-w protein.

Even yet another aspect there is provided a genetically modified animal comprising a mutation in one or more alleles of a gene comprising a sequence of nucleotides substantially
30 as set forth in SEQ ID NO:1 or SEQ ID NO:3 or a nucleotide sequence having at least about 47% similarity thereto and/or a sequence which is capable of hybridising to SEQ ID NO:1 or

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SEQ ID NO:3 under low stringency conditions at 42°C.

Even still another aspect of the present invention contemplates a method of producing a genetically modified animal substantially incapable of producing Bcl-w, said method

5 comprising introducing a genetic sequence into embryonic stem (ES) cells, which genetic sequence targets the *bcl-w* gene or a gene associate with *bcl-w* and introducing said ES cells into blastocysts to produce chimeric mice.

Another aspect of the present invention contemplates transgenic animals such as mice

10 containing a genetic sequence operably linked to a testis-specific promoter, which genetic sequence is capable of disrupting the *bcl-w* gene or *bcl-w* gene expression or expression of a gene associated with *bcl-w* in the testis.

Yet another aspect of the present invention is directed to a modified animal comprising a

15 mutation in a gene corresponding to *bcl-w* or a derivative or homologue thereof or in a gene associated with *bcl-w* wherein an adult male of said animal exhibits the following characteristics:

- (i) is substantially infertile;
- 20 (ii) possesses disorganised seminiferous tubules;
- (iii) exhibits heterogenous degeneration of germ cell types; and
- (iv) possesses no other major abnormalities as determined by histological examination.

Still yet a further aspect of the present invention contemplates an animal model for studying

25 other degenerative disorders such as but not limited to neurodegenerative disorders.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the disruption of the *bcl-w* gene. (A) The targeting vector *pbcl-wlox neo' tk*. Shaded bars represent regions derived from the *bcl-w* gene; *tk*, a thymidine kinase expression cassette; *neo'*, a PGK-*neo'* expression cassette; and diamonds, *loxP* sequences. (B) The wt *bcl-w* locus. Boxes represent exons (solid, coding region; open, untranslated region). E, *Eco* RI sites; sizes of *Eco* RI fragments are in kb. The *bcl-w* genomic DNA probes used for Southern blot analyses are labelled *a* and *b*, while the *bcl-w* cDNA sequences used as riboprobes are indicated by *c* and *d*. (C) Homologous recombination replaces the first 413 bp of the *bcl-w* coding region with a PGK-*neo'* expression cassette bounded by *loxP* sites. (D) Cre-mediated recombination deletes the PGK-*neo'* sequence, leaving only 127 bp of exogenous sequence, including a single *loxP* site. (E) Southern blot of genomic DNA from wt (+/+), heterozygous (+/-) and homozygous mutant (-/-) *bcl-w* mice (line 228), hybridized with *bcl-w* cDNA probe *a*. (F) Southern blot of genomic DNA from heterozygous mice (line 228) before (+/-) and after (+/ Δ) the action of Cre recombinase, hybridized with *bcl-w* probe *b*.

Figure 2 is a photographic representation showing expression of the *bcl-w* gene. (A) Northern blot of total RNA (10 μ g) extracted from the testes of 4-wk old wt (+/+) and *bcl-w^{ΔΔ}* mice (Δ/Δ), hybridized to a probe containing the first 1.2 kb of the *bcl-w* cDNA (upper panel); glyceraldehyde phosphate dehydrogenase mRNA served as a control (*gapdh*, lower panel). (B) Western blot analysis of protein lysates from the brain, testis and pancreas of wt and *bcl-w^{ΔΔ}* mice, using a polyclonal anti-Bcl-w antibody. The 21-kDa Bcl-w protein is indicated. (C) Western blots of protein lysates from testis cell lines, with the same antibody. GC-1 is a germ cell line derived from type B spermatogonia, TM4 a Sertoli cell line and TM3 a Leydig cell line; all were obtained from the American Type Culture Collection.

Figure 3 is a graphical representation showing reduced numbers of various cell types within the seminiferous tubules of *bcl-w^{ΔΔ}* mice. Frequencies of the indicated cell types was determined by the optical disector method for seven 6 wk-old wt mice and eight 6 wk-old *bcl-w^{ΔΔ}* mice. The percentage of the wt cell numbers remaining in the testes of *bcl-w^{ΔΔ}* mice is indicated. Error bars denote 2 standard errors of the means (SEM).

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Figure 4 is a graphical representation showing degeneration of testis in *bcl-w^{ΔΔ}* mice. (A) Mean mass of testes (3 mice per group). (B) TUNEL-labelled nuclei per tubule, counted at 2, 4, 8 and 14 wk (3 mice per group). Error bars denote 2 SEM.

5 Figure 5 is a diagrammatic representation of the consequences of *Bcl-w* loss in the testis. The percentages of the Sertoli cells and the different types of germ cells remaining in *bcl-w^{ΔΔ}* mice are indicated. The expression pattern of the gene is indicated schematically; the broken line indicates that the extent of expression in late stages of germ cell development remains to be clarified.

10

The following abbreviations are used in the subject specification.

B6	Mouse strain C57Bl/65
Cre	Cre recombinase
15 CSF	Colony-stimulating factor
ES	Embryonic stem
FSH	Follicle-stimulation hormone
G-CSF	Granulocyte Colony-stimulating factor
GM-CSF	Granulocyte-Macrophage Colony stimulating factor
20 LH	Lutenising hormone
M-CSF	Macrophage Colony-stimulating factor
<i>neo^r</i>	Neomycin phosphotransferase gene conferring resistance to neomycin
PBS	Phosphate buffered saline
PGK	Phosphoglycerate kinase
25 SDS-PAGE	Sodium diodecyl sulphate
<i>tk</i>	Thymidine kinase
TUNEL	Terminal transferase-mediated dUTP nick-end labelling
wk	Week
wt	Wild type.

30

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides a modified animal or avian species exhibiting reduced levels of a Bcl-w protein and/or a protein associated with Bcl-w or a derivative or homologue thereof, wherein said animal or avian species has an incapacity or a reduced capacity to induce or
5 facilitate spermatogenesis.

Reference herein to a "Bcl-w" protein includes reference to a protein having an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:4 or an amino acid sequence having approximately 47% or greater similarity to either of SEQ ID NO:2 or SEQ
10 ID NO:4. The nucleotide sequence set forth in SEQ ID NO:1 represents the human *bcl-w* gene while SEQ ID NO:3 is the murine *bcl-w* gene. The present invention extends, therefore, to Bcl-w with an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:4 as well as homologues, analogues or derivatives having at least about 47% similarity to the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:4. The Bcl-w protein or
15 its homologues or derivatives are encoded by a nucleotide sequence substantially as set forth in SEQ ID NO:1 (human) or SEQ ID NO:3 (murine) or a nucleotide sequence having at least 47% similarity thereto and/or which is capable of hybridising thereto under low stringency conditions at 42°C. All such derivatives and homologues are encompassed by the terms "Bcl-w" (for the protein) or "*bcl-w*" (for the nucleic acid). Examples of derivatives of *bcl-w*
20 include the nucleotide sequence set forth in SEQ ID NO:5 (human) or SEQ ID NO:7 (murine) or their corresponding amino acid sequences (SEQ ID NO:6 and SEQ ID NO:8, respectively). Wild type *bcl-w* may also be defined by reference to a nucleotide sequence capable of hybridising to a derivative of SEQ ID NO: 1 or SEQ ID NO:3, such as SEQ ID NO:5 or SEQ ID NO:7.

25

Accordingly, another aspect of the present invention provides a modified animal or avian species exhibiting reduced levels of a Bcl-w protein having an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:4 or a Bcl-w protein encoded by a nucleotide sequence substantially set forth in SEQ ID NO:1 or SEQ ID NO:3 or a nucleotide
30 sequence capable of hybridising to SEQ ID NO:1 or 3 or 5 or 7 under low stringency conditions at 42 °C wherein said animal or avian species has an incapacity or a reduced

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capacity to induce or facilitate spermatogenesis.

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, 5 "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particularly preferred embodiment, nucleotide 10 and sequence comparisons are made at the level of identity rather than similarity. Any number of programs are available to compare nucleotide and amino acid sequences. Preferred programs have regard to an appropriate alignment. One such program is Gap which considers all possible alignment and gap positions and creates an alignment with the largest number of matched bases and the fewest gaps. Gap uses the alignment method of 15 Needleman and Wunsch (20). Gap reads a scoring matrix that contains values for every possible GCG symbol match. GAP is available on ANGIS (Australian National Genomic Information Service) at website <http://mell.angis.org.au..>

Reference herein to a low stringency at 42°C includes and encompasses from at least about 20 1% v/v to at least about 15% v/v formamide and from at least about 1M to at least about 2M salt for hybridisation, and at least about 1M to at least about 2M salt for washing conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5M to at least about 0.9M salt for 25 hybridisation, and at least about 0.5M to at least about 0.9M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01M to at least about 0.15M salt for hybridisation, and at least about 0.01M to at least about 0.15M salt for washing conditions.

30 Preferably, the percentage similarity or identity at the amino acid or nucleotide levels is between 48% and 100% inclusive such as approximately 50% or 55%, 59% or 65%, 70% or

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75%, 80% or 85%, 90% or 95% or greater than 96% or a percentage similarity or identity there between.

A gene associated with *bcl-w* or a protein associated with Bcl-w includes the gene which is approximately 9.2 kb down stream of *bcl-w* exon 3 and which has homology to the *Drosophila rox* gene (13). Fusion RNA transcripts have been observed between *bcl-w* and *rox* and, hence, disruption of the *rox* gene or its transcript or translation production may impact on *bcl-w* expression or Bcl-w activity. The present invention extends, therefore, to targeting Rox, *rox*, *bcl-w-rox* fusion transcripts and Bcl-w-Rox fusion translation products.

10 The present invention extends to other genes associate with *bcl-w* at the regulation, transcription or proximity levels.

Preferably, the Bcl-w protein is of mammalian origin such as from humans, primates, livestock animals (eg. sheep, cows, horses, pigs), companion animals (eg. cats, dogs), 15 laboratory test animals (eg. rabbits, mice, rats, guinea pigs) and captive wild animals (eg. foxcs, deer, kangaroos). However, the present invention also extends to non-mammalian homologues of Bcl-w such as from avian species, fish and reptiles. Generally, when producing a modified animal, the effector molecules to reduce Bcl-w activity or expression are identified on the basis of a Bcl-w from the same species. However, an effector molecule 20 against, for example, murine Bcl-w may also be used against human Bcl-w. Both types of effector molecules are contemplated by the present invention and are referred to as heterologous or homologous effector molecules. Similar comments apply with respect to a gene associated with *bcl-w* or a protein associated with Bcl-w.

25 According to a particularly preferred embodiment, there is provided a modified animal exhibiting reduced levels of Bcl-w or a derivative or homologue thereof and/or of a protein associated with Bcl-w wherein said Bcl-w or its derivative or homologue comprises an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:4 or an amino acid sequence having at least about 47% similarity to the amino acid sequence of SEQ ID NO:2 or 30 SEQ ID NO:4 and wherein said modified animal has a incapacity or a reduced capacity to induce or facilitate productive spermatogenesis.

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In a related embodiment, there is provided a modified animal exhibiting reduced levels of Bcl-w or a derivative or homologue thereof and/or of a protein associated with Bcl-w wherein said Bcl-w or its derivative or homologue is encoded by a nucleotide sequence substantially as set forth in SEQ ID NO:1 or SEQ ID NO:3 or a nucleotide sequence having at least 47% similarity thereto and/or which can hybridise to SEQ ID NO:1 or SEQ ID NO:3 under low stringency conditions at 42°C.

The "modified" animal may be modified at the level of Bcl-w family protein activity or at the genetic level of the *bcl-w* gene. In regards to the former, the present invention contemplates the administration of a range of antagonists to Bcl-w protein activity resulting in reduced or substantially total removal of Bcl-w protein activity. For example, a vaccine may be administered containing Bcl-w protein or an immunogenic derivative thereof to induce antibodies to endogenous Bcl-w protein. Alternatively, a molecule identified from natural product screening capable of acting as an antagonist may be employed. Due to the intracellular nature of Bcl-w, antagonists are generally small molecules or in a form capable of entry into cells. A particularly important potential antagonist is a molecule containing a BH3 amino acid motif. The term "BH" stems from "Bcl-2 Homology" and relates to regions of homology between Bcl-2 proteins (reviewed by Kroemer (8)). The BH3 domain is capable of binding to Bcl-2 and related molecules. Accordingly, a small molecule, for example, a peptide comprising a BH3 motif or closely related to it, or a chemical mimetic thereof may provide antagonist activity towards Bcl-w. Similar considerations apply in respect of a gene or protein associated with *bcl-w* or Bcl-w, respectively.

The present invention further contemplates the use of naturally occurring molecules such as Bim (37) to regulate Bcl-w activity. Such molecules interact or otherwise associate with Bcl-w activity. Such molecules interact or otherwise associate with Bcl-w to modulate its activity.

The present invention further contemplates genetic vaccinations. For example, a DNA vaccine may be prepared in order to induce an immune response against Bcl-w. Enhanced immunogenicity may be obtained using molecular adjuvants such as a peptide derived from

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the C3d region which binds to the CR2 receptors on B cells (21). Other suitable molecule adjuvants include L. selectin and cytotoxic T-lymphocyte antigen (CTLA4) (22) or CD40 (23).

5 According to another aspect of the present invention there is provided a modified animal exhibiting an incapacity or a reduced capacity to induce or facilitate productive spermatogenesis said modification comprising the administration to said animal of an antagonistic effective amount of a molecule capable directly or indirectly of antagonising Bcl-w protein activity or the ability of a derivative or homologue of Bcl-w.

10

Examples of molecules directly affecting Bcl-w protein activity include an antibody, a soluble receptor for Bcl-w protein and a chemical found from natural product screening or the screening of synthetic libraries. An example of a molecule indirectly affect Bcl-w family protein activity includes a Bcl-w protein or an immunogenic derivative thereof capable of
15 inducing an immune response against an endogenous Bcl-w protein. Another example is a molecule which targets a gene or protein associated with *bcl-w*/Bcl-w. As stated above, these molecules may need to be modified to permit entry into target cells.

In a related embodiment, there is provided a composition capable of inducing infertility or
20 reducing fertility in an animal, said composition comprising a direct or indirect antagonist of a Bcl-w protein.

Reference to "natural product screening" includes products identified from sources such as but not limited to coral, soil, seabeds and sea water, bacteria, yeasts, plants and river water
25 and river beds.

The composition of this aspect of the present invention may also comprise one or more carriers and/or diluents. Preferably the carriers are pharmaceutically acceptable.

30 The target animals are as stated above such as humans, primates, livestock animals, laboratory test animals and companion animals. The preferred modified animal, however, for

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the purposes of an *in vivo* model is a mouse, rat, rabbit, guinea pig, sheep or pig. The most preferred animal is a mouse.

Another aspect of the present invention relates to the genetic reduction in Bcl-w protein
5 levels. According to this aspect of the present invention, there is provided a genetically modified animal comprising a mutation in one or more alleles of a gene encoding a Bcl-w protein and/or of a gene associated with Bcl-w protein.

In a related embodiment, there is provided a genetically modified animal comprising a
10 mutation in one or more alleles of a gene comprising a sequence of nucleotides substantially as set forth in SEQ ID NO:1 or SEQ ID NO:3 or a nucleotide sequence having at least about 47% similarity thereto and/or a sequence which is capable of hybridising to SEQ ID NO:1 or SEQ ID NO:3 under low stringency conditions at 42°C.

15 Preferably, in order to observe the infertility phenotype, the animal model comprises an animal with a mutation in both alleles of *bcl-w* and is referred to as "*bcl-w*^{ΔΔ}" which is considered equivalent to the designation "*bcl-w*^{-/-}". An animal with a mutation in one copy of the gene is referred to as "*bcl-w*^{+Δ}" or "*bcl-w*^{+/-}". A *bcl-w*^{+Δ} animal is also useful as a carrier for the *bcl-w*^{ΔΔ} genotype. Reference to a *bcl-w*^Δ genotype is not to imply deletion of the
20 entire coding region for Bcl-w although such a deletion is contemplated by the present invention. Partial deletion or any nucleotide insertion, deletion and/or addition is encompassed by the term "*bcl-w*^{ΔΔ}" or "*bcl-w*^{Δ+}".

In accordance with the present invention, animals and in particular mice carrying a mutation
25 in the *bcl-w* gene have normal populations of lymphoid, myeloid and erythroid cells in bone marrow, spleen, thymus and peripheral blood and normal numbers of haematopoietic progenitors in bone marrow. Adult female *bcl-w*^{ΔΔ} mice are fertile. However, adult male *bcl-w*^{ΔΔ} mice are infertile and have small testes. There are no other major abnormalities as determined by, for example, histological examination. The *bcl-w*^{ΔΔ} mice grow more slowly
30 after puberty than wild-type littermates. The structure of the seminiferous tubules of adult *bcl-w*^{ΔΔ} mice is disorganised and the tubules are difficult to categorise according to the

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normal spermatogenic cycle. Heterogeneous degeneration of all germ cell types is evident, with some degenerating giant cells visible in the tubule lumen. While some round spermatids are present, there are few metamorphosing spermatids and no mature sperm. Seminiferous tubules of *bcl-w*^{ΔΔ} mice contain increased numbers of apoptotic nuclei which label with the
5 TUNEL technique, compared to tubules of wild-type littermates. The testes of 2 week old and 4 week old *bcl-w*^{ΔΔ} mice appear grossly normal and contain some metamorphosing spermatids.

The term "mutation" is used in its broadest sense and includes a single or multiple nucleotide
10 substitution, deletion and/or addition to *bcl-w* or to a region controlling *bcl-w* expression such as a promoter, polyadenylation signal or regulatory gene. The mutation generally results in no active Bcl-w protein being produced or substantially reduced levels of Bcl-w protein being produced. The mutation may also involve a splice variant. The mutation may also be outside the *bcl-w* gene but in a gene associated with *bcl-w* such as the *rox* gene. The term
15 *bcl-w*^{ΔΔ} denotes the absence of a functional Bcl-w protein. For convenience, it is also used to cover reduced levels of functional Bcl-w such as in the case of the administration of an antagonist of Bcl-w or if antisense molecules are used to induce a transient reduction in Bcl-w levels.

20 In a particularly preferred embodiment, a substantial portion of the gene has been deleted through, for example, homologous recombination. One particularly useful method is depicted in Figure 1. According to this preferred method a plasmid targeting vector is prepared (eg. denoted *lox-neo bcl-w*) and transfected into embryonic stem (ES) cells. ES cell lines carrying one copy of the targeted *bcl-w* locus are generated and injected into blastocysts to produce
25 chimeric mice. A targeting vector is preferably designed to replace almost the entire *bcl-w* coding sequence with a *pgk-neo* expression cassette. The *pgk-neo* cassette is bounded by sites (*loxP*) that allow its subsequent excision by the action of the bacteriophage Cre recombinase. In order to achieve this, chimeric mice carrying the *bcl-w* mutation have been bred with mice expressing a *Cre* transgene. The correct disruption of the *bcl-w* locus by
30 homologous recombination and removal of the selectable marker by Cre-mediated recombination is confirmed by polymerase chain reaction and Southern blotting. Subsequent

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breeding generates *bcl-w*^{ΔΔ} mice. A similar approach can be used to mutate a gene associated with *bcl-w*.

There are a number of other mechanisms for generating *bcl-w*^{ΔΔ} mice or *bcl-w*^{+/Δ} mice and all
5 these are encompassed by the present invention.

In addition, the present invention further contemplates transient disruption of the *bcl-w* gene through use of antisense molecules, ribozymes and deoxyribozymes. Viruses may also be employed to introduce antisense molecules or other molecules capable of disrupting function
10 of the *bcl-w* gene. All such genetic molecules are encompassed by the present invention.

Another aspect of the present invention contemplates a method of producing a genetically modified animal substantially incapable of producing Bcl-w, said method comprising introducing a genetic sequence into ES cells, which genetic sequence targets the *bcl-w* gene
15 or a gene associate with *bcl-w* and introducing said ES cells into blastocysts to produce chimeric mice.

The genetic sequence permits excision of the *bcl-w* gene or a selectable marker or specific region within or associated with the *bcl-w* gene by, for example, Cre recombinase.
20

Preferably, the animal is a mouse.

The ES cells may be from the recipient animal (allergenic) or from a different animal of the same species (heterogenic).
25

The modified animals of the present invention are particularly useful in screening for genetic or non-genetic molecules capable of restoring fertility. They are also useful as a model for studying the effects of infertility and in the rationale design of molecules capable of inducing infertility.

30

The *bcl-w*^{ΔΔ} mutation may also be linked to a "reporter" gene, such as could be used to

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illustrate expression of *bcl-w* in adult male mice and/or in mouse embryos. For breeding and screening purposes, such a readily identifiable marker would greatly facilitate the identification of *bcl-w*^{ΔΔ} mice.

- 5 Agonists and antagonists of *bcl-w* or Bcl-w are also readily obtained by screening for molecules capable of interacting with the protein or modifying *bcl-w* expression. One useful assay involves culturing cells which are *bcl-w*^{+/+} or *bcl-w*^{ΔΔ} and adding potential modulators and screens for apoptosis or reversal of apoptosis.
- 10 A further embodiment of the present invention contemplates transgenic animals such as mice containing a genetic sequence operably linked to a testis-specific promoter, which genetic sequence is capable of disrupting the *bcl-w* gene or *bcl-w* gene expression or expression of a gene associated with *bcl-w* in the testis.
- 15 Yet a further embodiment of the present invention is directed to a modified animal comprising a mutation in a gene corresponding to *bcl-w* or a derivative or homologue thereof or in a gene associated with *bcl-w* wherein an adult male of said animal exhibits the following characteristics:
- 20 (i) is substantially infertile;
(ii) possesses disorganised seminiferous tubules;
(iii) exhibits heterogenous degeneration of germ cell types; and
(iv) possesses no other major abnormalities as determined by histological examination.
- 25 In murine and human species the *bcl-w* mutation is on chromosome 14 and specifically 14q11 in humans. It may be located on other chromosomes in other species.

Yet a further embodiment of the present invention contemplates an animal model for studying other degenerative disorders such as but not limited to neurodegenerative disorders. For
30 example, animals such as mice which are *bcl-w*^{+/Δ} or *bcl-w*^{ΔΔ} in glial cells may ultimately develop a neurodegenerative disorder. Such animal models would be useful in screening for

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The present invention is further described by the following non-limiting Examples.

Examples 1 to 9 provide the materials and methods employed to obtain the data of Example 10.

5

EXAMPLE 1 DISRUPTION OF *bcl-w*

The *bcl-w* gene was inactivated by homologous recombination. The gene targeting vector (see Fig. 1A) was assembled in *ploxPneo-1* in which a neomycin phosphotransferase gene (*neo'*), driven by a phosphoglycerate kinase (PGK) promoter, is flanked by bacteriophage P1 *loxP* sites. The 129/Sv mouse *bcl-w* genomic DNA sequences introduced at each end of the *loxP-neo'-loxP* cassette comprised the 876 bp region immediately upstream of the *bcl-w* start codon and the 4-kb *Bam* HI fragment extending from within exon 3 through the entire 3' untranslated region. Introduction of a terminal herpes simplex virus thymidine kinase (*tk*) gene driven by a PGK promoter then completed the vector (Fig. 1A), which was linearized and electroporated into W9.5 ES cells (24). ES cell clones selected for resistance to G418 (i.e. *neo'* gene integration) and gancyclovir (i.e. loss of the *tk* gene following homologous recombination) (25) were screened for homologous recombination at the *bcl-w* locus by Southern blot analysis. The *bcl-w* mutant ES cell clones were injected into the blastocoel cavity of C57BL/6J (B6) blastocysts, which were then implanted into pseudopregnant foster mothers. Male chimeric progeny were crossed to B6 females or, to delete the *neo'* cassette, to B6/FVB F1 females expressing bacteriophage P1 Cre recombinase (Cre) (26).

25

EXAMPLE 2 ANALYSIS OF MOUSE WEIGHTS

Wild type (wt) and mutant mice were weighed weekly from birth to 20 wk, and the weights analyzed using the split-line model (27). Briefly, growth curves before and after puberty were fitted to two straight lines, and the slopes of these lines and their point of intersection compared.

30

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EXAMPLE 3

BLOT ANALYSIS

Southern blot analysis on cultured ES cells or mouse tail tips used 500-bp *Stu* I-*Bam* HI and
5 4-kb *Pml* I genomic DNA fragments (probes *a* and *b* respectively in Fig 1B). Northern blot
analysis was conducted on total RNA (10 μ g/lane) prepared (28) from testes of adult mice.
For western blot analysis, tissues or cells were washed in phosphate-buffered saline (PBS),
immediately frozen in isopentane on dry ice, homogenized at 4 °C in buffer (50 mM TrisHCl
(pH 7.5), 2 mM EDTA, 1% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride, 2
10 μ g/ml aprotinin, 1 μ g/ml pepstatin and 2 μ g/ml leupeptin and then centrifuged at 10,000 \times g
at 4 °C for 30 min. Proteins (35 μ g) in the supernatant were resolved by SDS-PAGE (12%
w/v acrylamide gel) and transferred to nitrocellulose membranes (Hybond-C extra,
Amersham). As controls for protein loading and integrity, membranes were stained with
Ponceau S, or with an antibody against the ubiquitous Hsp-70. Bcl-w was detected by
15 incubation of the membranes overnight with a polyclonal rabbit-anti-human Bcl-w antibody
(AAP-050, StressGen Biotechnologies), followed by horseradish peroxidase-conjugated goat
anti-rabbit antibody (Selenius) and chemiluminescent reagents (Amersham).

EXAMPLE 4

HISTOLOGY AND BrdUrd LABELLING

20 Tissues fixed in Bouin's solution for 5 hr were embedded in paraffin, and 8 μ m sections
transferred to silane-coated microscope slides and stained with hematoxylin and eosin. The
following tissues were examined: brain, colon, salivary gland, liver, heart, stomach, skeletal
25 muscle, skin, peripheral nerve, pituitary gland, eye, teeth, bone, cartilage, thyroid and
parathyroid glands, blood vessels, lung, small intestine, pancreas, kidney, adrenal gland,
bladder, uterus, ovary and testis. To determine mitotic turnover, mice were injected i.p. with
BrdUrd (100 μ g/g body weight in 7 mM NaOH) 8 hr before sacrifice. Paraffin-embedded
sections of testis, small intestine, colon, spleen, thymus and bone marrow were stained with
30 rat-anti-BrdUrd antibody (Mas 250P, Harlan Ser-Lab). This was detected by biotinylated
mouse-anti-rat Igk antibody (Mar 18.5), avidin-biotinylated horseradish peroxidase (Elite

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ABC, Vector Laboratories) and diaminobenzidine.

EXAMPLE 5

TERMINAL TRANSFERASE-MEDIATED dUTP NICK END-LABELLING (TUNEL)

Paraffin-embedded sections were treated with 20 $\mu\text{g/ml}$ proteinase K in water for 15 min at room temperature, then DNA free ends were labelled with dUTP-biotin using terminal deoxynucleotidyl transferase (29) and revealed with avidin-biotinylated horseradish
10 peroxidase. For each testis, TUNEL-labelled (apoptotic) nuclei in approximately twenty-five 0.56 mm² fields were counted, and the number of apoptotic nuclei per seminiferous tubule determined.

EXAMPLE 6

HEMATOLOGIC ANALYSIS

Peripheral blood erythrocytes and leucocytes were enumerated using a Coulter counter, and platelets with a Sysmex NE8000 counter (TOA, Kobe, Japan). Leucocytes in peripheral blood, femoral bone marrow, peritoneum, spleen and thymus were stained with eosin and
20 counted by hemocytometer. Cytocentrifuge preparations were stained with May-Grunwald-Giemsa. Single cell suspensions prepared from blood, bone marrow, spleen and thymus were incubated with 2.4G2 anti-Fc γ receptor antibody (30) to reduce background staining, labeled with fluorescent surface marker-specific monoclonal antibodies and analysed by flow cytometry as elsewhere described (31).

25

To enumerate progenitor cells, bone marrow and spleen cells were cultured in medium containing 0.1% w/v agar (32) and the following cytokines: 10 ng/ml murine granulocyte-macrophage-colony stimulating factor (GM-CSF), 10 ng/ml human granulocyte-CSF (G-CSF), 10 ng/ml murine macrophage-CSF (M-CSF), 10 ng/ml murine interleukin-3, 100 ng/ml
30 murine stem cell factor or 200 ng/ml murine thrombopoietin. To determine the cellular composition of each colony, the agar plates were fixed and stained for acetylcholinesterase,

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then with Luxol fast blue and hematoxylin (32).

EXAMPLE 7 TESTIS STEREOLOGY

5

Testes fixed for 5 hr in Bouin's fixative were embedded in methacrylate; 25 μ m sections were transferred to glass slides and stained with hematoxylin and the periodic acid-Schiff reagent. Leydig and Sertoli cells and germ cells were counted using the 'optical disector' approach as described previously (33).

10

EXAMPLE 8 IN SITU HYBRIDISATION

Digoxigenin-labelled riboprobes were generated from linearized plasmid DNA templates (34).
15 Riboprobes *c1* (sense) and *c2* (anti-sense) (Fig. 1B) were generated from residues 118 to 410 of the *bcl-w* cDNA (GenBank U59746) in the pT7Blue vector (Novagen), and *d1* and *d2* from residues 330 to 956 in the pBSIISK vector (Stratagene). Paraffin-embedded tissue sections on microscope slides were treated with 1 μ g/ml proteinase K in buffered saline for 30 min at 37 °C, hybridized to the riboprobes at 50 °C for 16 hr, and washed to 0.1 x SSC at
20 50 °C (34). Slides were then exposed to an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim), riboprobes detected with the nitroblue tetrazolium chloride / bromo-chloro-indolyl phosphate substrate, and the slides counterstained with hematoxylin.

25

EXAMPLE 9 SERUM GONADOTROPHIN ASSAY

The concentration of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in serum was determined by a double-antibody radioimmunoassay using reagents for the
30 measurement of rat FSH and LH (35). Their efficacy on the mouse hormones was confirmed. All samples were measured in the same assay with an intra-assay coefficient of variation of

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4.8% and 5.9% for the FSH and LH assays, respectively.

EXAMPLE 10

RESULTS

5 Disruption of *bcl-w*

The gene targeting vector was designed to inactivate *bcl-w* by replacing the first two thirds of its coding region with a PGK-*neo'* expression cassette bounded by *loxP* sites (Fig. 1A-C).

Any translation of the remainder should be precluded by a preceding stop codon.

Homologous recombination was obtained in 8 of 352 selected ES cell clones. The structure

10 of the mutant allele (*bcl-w'*) was confirmed by Southern blot analysis: *bcl-w* probe *a* detected 6.6-kb and 5.0-kb *Eco* RI fragments diagnostic for the wt and *bcl-w'* alleles, respectively (e.g. Fig. 1E). A *neo'* probe excluded the presence of any copies of the targeting vector integrated elsewhere in the genome. Two independent recombinant ES clones were used to generate chimeric mice, which were bred with B6 females to generate two lines of *bcl-w-*
15 mutant mice (228 and 229), each of which was subsequently bred to homozygosity.

Regulatory sequences introduced by gene targeting can inadvertently alter the expression of neighbouring genes. Just 5.5 kb downstream of *bcl-w* is the gene encoding poly (A)-binding protein II (*mPABII* (36), homologue of *rox* (19)). To avoid altering the expression of this or
20 other neighboring genes, the inventors also generated mice in which the introduced PGK-*neo'* cassette was deleted by crossing both 228 and 229 mice with animals expressing Cre recombinase at the 2-cell stage of development (3) (Fig. 1D). Progeny carrying the deleted allele (*bcl-w^Δ*, Fig. 1D) were recognized by a diagnostic 1.1-kb *Eco* RI fragment (Fig. 1F), and the deletion was confirmed by sequencing a PCR product spanning the recombination
25 site. Crosses with B6 mice then generated lines 228Δ and 229Δ. Northern blot analysis confirmed that expression of the *mPABII* gene was unaffected in 228Δ mice homozygous for the *bcl-w^Δ* allele. Importantly, homozygous mutants of all four lines (228, 229, 228Δ and 229Δ) proved to be indistinguishable.

30 *Bcl-w* is dispensable for development

As expected, the *bcl-w^{Δ/Δ}* mice expressed neither *bcl-w* RNA nor protein. No RNA transcript

was detected by a *bcl-w* cDNA probe in northern blots of RNA extracted from testis (Fig. 2A), and western blots with an anti-Bcl-w antibody revealed no Bcl-w protein in lysates from brain, testis or pancreas (Fig. 2B).

- 5 Lack of Bcl-w did not compromise survival of fetal or neonatal mice. The offspring of *bcl-w*^{+/+} intercrosses were born at normal Mendelian frequency: 25% *bcl-w*^{+/+}, 47% *bcl-w*^{+/-} and 28% *bcl-w*^{-/-}, and 57% of *bcl-w*^{-/-} offspring were male (n total = 210). The *bcl-w*^{-/-} mice exhibited no significant abnormality in external appearance or behavior. The growth of *bcl-w*^{-/-} pups from birth to 5 wk of age was indistinguishable from that of their wt littermates.
- 10 Although the average weights of male and female *bcl-w*^{-/-} mice at 5, 7, 9, 12, 16 and 20 wk of age were slightly less than that of their *bcl-w*^{+/+} and *bcl-w*^{+/-} littermates, the differences were not statistically significant. In addition, the growth curves of wt and *bcl-w*^{-/-} mice were indistinguishable when analyzed using the split-line method (27). Thorough histological examination of numerous tissues (see Examples 1 to 9) from *bcl-w*^{-/-} mice 6 and 52 wk of age
- 15 revealed no significant abnormalities.

Normal maintenance of hematopoiesis

- Since *bcl-w* RNA is detectable in most myeloid and some lymphoid cell lines (19), the hematopoietic tissues of *bcl-w*^{-/-} mice were carefully scrutinized. In mice analyzed at 6 and
- 20 52 wks, the weight and histology of the thymus, spleen, lymph node and bone marrow were normal. Blood cell analysis of three adult mice indicated normal numbers of erythrocytes, platelets, neutrophils, monocytes, eosinophils and lymphocytes (B and T). The peritoneal leucocyte population was also unaffected. The frequency of apoptotic nuclei in the spleen, thymus and bone marrow was unaltered, as judged by TUNEL analysis (29). Bcl-2 family
 - 25 members can slow mitotic cycle entry, but immunohistochemistry of spleen, thymus and bone marrow from *bcl-w*^{-/-} mice injected with BrdUrd 8 hours before sacrifice (see Examples 1-9) indicated normal numbers of leucocytes in the S phase.

- Clonogenic assays on bone marrow cells from three adult *bcl-w*^{-/-} mice and three wt
- 30 littermates yielded a comparable frequency of neutrophil, neutrophil-macrophage, macrophage, eosinophil, megakaryocyte and blast cell colony-forming cells, and the colonies

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were of similar size and maturation. Moreover, the progenitors were not rendered more sensitive to cytokine deprivation, since a 4-day delay in addition of interleukin-3 to such cultures reduced the number of colonies from wt and mutant marrow to equivalent extents.

5 *Bcl-w* is essential for spermatogenesis

Female *bcl-w^{ΔΔ}* mice were fertile and competent to feed their pups. Intriguingly, however, all the males were infertile. While their external genitalia and testicular descent appeared normal, the cauda epididymides of *bcl-w^{ΔΔ}* mice of all ages were devoid of sperm. In contrast, male heterozygotes exhibited normal fertility and epididymal histology.

10

Spermatogenesis involves an orderly process of germ cell maturation towards the center of the seminiferous tubules: mitotic proliferation of spermatogonia (up to 9 divisions), meiotic division of spermatocytes, differentiation of spermatids and finally release of spermatozoa into the tubule lumen. Histological examination of the testes of adult *bcl-w^{ΔΔ}* mice revealed

15 extensive albeit heterogeneous pathology within the seminiferous tubules. The tubules were abnormally small in diameter and often lacked a lumen. Numerous degenerating cells appeared throughout the seminiferous epithelium, some in the form of symplasts, giant cells containing several degenerating nuclei. There were few elongating spermatids more advanced than stage 13 of the seminiferous cycle and no mature sperm. Indeed, by 52 wk of age,

20 almost no germ cells were discernible, although Sertoli cells remained. The defect was not in proliferation, since anti-BrdUrd-immunohistochemistry revealed numerous spermatocytes in S phase. Instead there was a striking elevation in the number of TUNEL-labelled apoptotic cells, many of which were contained within symplasts.

25 To determine which cells were affected, the inventors used the well-characterized 'optical disector' method (see Examples 1-9) to calculate the total number of each cell type within the testes of wt and *bcl-w^{ΔΔ}* mice at 6 wk of age. Leydig cells were increased by nearly 50%. For each of the other cell types analyzed, however, mutant testes contained significantly fewer cells than wt testes (Student's t-tests, $P < 0.05$). Sertoli cell numbers had decreased to 16% of

30 their normal level (Fig. 3). Interestingly, germ cell numbers declined progressively with advancing stages of differentiation. Whereas type A spermatogonia were 30% of the normal

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level, spermatocytes represented only 15% to 20% of normal numbers, and, during spermatid differentiation, the level fell to 3% of normal (Fig. 3). Cells were also enumerated in the testes of single wt and *bcl-w^{wt}* mice at 12, 14 and 16 wk of age. The deficit of round and elongating spermatids was more severe by 12 wk of age, and by 14 wk very few cells at or beyond the pachytene spermatocyte stage remained. Heterozygotes exhibited none of these alterations.

Germ cell apoptosis increases near sexual maturity

Early testicular development was normal. At 2wk of age, the testes of *bcl-w^{wt}* mice exhibited normal mass and histology, and the number of TUNEL-labelled apoptotic nuclei per tubule was similar to that of wt littermates (Fig. 4). Even at 4 wk, the testes appeared normal and were of normal weight (Fig. 4A), suggesting that germ cell numbers had not yet fallen substantially, although there were twice as many apoptotic cells as in wt littermates (Fig. 4B). By 8 wk of age, however, the number of apoptotic cells was 5 times the normal level, and the testes had lost 70% of their mass (Fig. 4). Subsequently, the frequency of apoptotic cells declined, probably because so few germ cells remained. Thus, the apoptotic loss commences by 4 wk of age but severe attrition is evident only at sexual maturity.

No evidence for an endocrinological basis

Germ cell apoptosis is inhibited directly by circulating androgens and FSH, and indirectly by LH, which promotes the secretion of androgens by Leydig cells (32, 18). It seemed possible, therefore, that the spermatogenic defect was caused by reduced levels of these hormones. However, normal androgen levels could be inferred from the unaltered weight and histology of androgen-dependent organs (ventral prostate gland and seminal vesicles). Moreover, the serum FSH and LH concentrations of six wt and six *bcl-w^{wt}* mice were equivalent (Student's t-test, $P = 1.0$ for FSH and 0.1 for LH). These results, together with the normal histological appearance of the Leydig cells, hypothalamus and pituitary gland, make it unlikely that altered endocrine levels have a major role in the phenotype.

Expression of *bcl-w* in the testis

To facilitate interpretation of the phenotype of *bcl-w^{ΔΔ}* mice, the inventors explored the expression pattern of *bcl-w* in wt adult testis. *In situ* hybridization indicated that *bcl-w* RNA was very prominent in the basal regions of seminiferous tubules. Antisense *bcl-w* riboprobes (*c1* and *d1*, Fig. 1B) hybridized strongly to spermatogonia and moderately to spermatocytes, round spermatids and some Sertoli cells, but not detectably to elongating spermatids or mature sperm. Corresponding sense riboprobes (*c2*, *d2*) did not hybridize to any cell type and the antisense probes failed to detect any cells in the testis of *bcl-w^{ΔΔ}* mice. Thus, *bcl-w* expression in adult testis was most conspicuous in pre-meiotic germ cells and was detectable in Sertoli cells but not in Leydig cells. The consequences of loss of Bcl-w in the testis is shown in Figure 5.

The expression profile of Bcl-w in three mouse testicular cell lines was in accord with the *in situ* hybridization. Western blot analysis with a polyclonal anti-Bcl-w antibody revealed high levels of Bcl-w protein in the germ cell line GC-1 (derived from type B spermatogonia) and moderate levels in the Sertoli cell line TM4, but none in the Leydig line TM3 (Fig. 2C). Bcl-w was also detected in testes of 10-day old mice, which contain only Sertoli cells and spermatogonia.

Summary

Proteins of the Bcl-2 family are important regulators of apoptosis in many tissues of the embryo and adult. The recently isolated *bcl-w* gene encodes a novel pro-survival member of the Bcl-2 family which is widely expressed. To explore its physiological role, the inventors inactivated the *bcl-w* gene in the mouse by homologous recombination. Mice which lack Bcl-w were viable, healthy and normal in appearance. Most tissues exhibited typical histology, and hematopoiesis was unaffected, presumably due to redundant function with other pro-survival family members. While female reproductive function was normal, the males were infertile. The testes developed normally and the initial, prepubertal wave of spermatogenesis was largely unaffected. The seminiferous tubules of adult males, however, were disorganized, contained numerous apoptotic cells and produced no mature sperm. Both Sertoli cells and germ cells of all types were reduced in number, the most mature germ cells being the most severely depleted. The *bcl-w^{ΔΔ}* mouse provides a unique model of failed spermatogenesis in the adult which has relevance to aspects of human male sterility.

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(C) TELEX: AA 31787

$\begin{array}{l} \text{H}_2\text{N}-[\text{CH}_2-\text{C}(=\text{O})]_n-\text{NH}_2 \\ n=6-8 \end{array}$

[illegible][illegible][illegible][illegible][illegible][illegible][illegible][illegible][illegible][illegible][illegible]

- 33 -

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 193 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 1              5              10              15
Phe Val Gly Tyr Lys Leu Arg Gln Lys Gly Tyr Val Cys Gly Ala Gly
      20              25              30
Pro Gly Glu Gly Pro Ala Ala Asp Pro Leu His Gln Ala Met Arg Ala
      35              40              45
Ala Gly Asp Glu Phe Glu Thr Arg Phe Arg Arg Thr Phe Ser Asp Leu
 50              55              60
Ala Ala Gln Leu His Val Thr Pro Gly Ser Ala Gln Gln Arg Phe Thr
 65              70              75              80
Gln Val Ser Asp Glu Leu Phe Gln Gly Gly Pro Asn Trp Gly Arg Leu
      85              90              95
Val Ala Phe Phe Val Phe Gly Ala Ala Leu Cys Ala Glu Ser Val Asn
      100             105             110
Lys Glu Met Glu Pro Leu Val Gly Gln Val Gln Glu Trp Met Val Ala
      115             120             125
Tyr Leu Glu Thr Arg Leu Ala Asp Trp Ile His Ser Ser Gly Gly Trp
      130             135             140
Ala Glu Phe Thr Ala Leu Tyr Gly Asp Gly Ala Leu Glu Glu Ala Arg
      145             150             155             160
Arg Leu Arg Glu Gly Asn Trp Ala Ser Val Arg Thr Val Leu Thr Gly
      165             170             175
Ala Val Ala Leu Gly Ala Leu Val Thr Val Gly Ala Phe Phe Ala Ser
      180             185             190
Lys

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 582 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..582

[illegible]

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 193 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Met Ala Thr Pro Ala Ser Thr Pro Asp Thr Arg Ala Leu Val Ala Asp
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Phe Val Gly Tyr Lys Leu Arg Gln Lys Gly Tyr Val Cys Gly Ala Gly
      20             25             30
Pro Gly Glu Gly Pro Ala Ala Asp Pro Leu His Gln Ala Met Arg Ala
      35             40             45
Ala Gly Asp Glu Phe Glu Thr Arg Phe Arg Arg Thr Phe Ser Asp Leu
      50             55             60
Ala Ala Gln Leu His Val Thr Pro Gly Ser Ala Gln Gln Arg Phe Thr
      65             70             75             80
Gln Val Ser Asp Glu Leu Phe Gln Gly Gly Pro Asn Trp Gly Arg Leu
      85             90             95
Val Ala Phe Phe Val Phe Gly Ala Ala Leu Cys Ala Glu Ser Val Asn
      100            105            110
Lys Glu Met Glu Pro Leu Val Gly Gln Val Gln Asp Trp Met Val Ala
      115            120            125
Tyr Leu Glu Thr Arg Leu Ala Asp Trp Ile His Ser Ser Gly Gly Trp
      130            135            140
Ala Glu Phe Thr Ala Leu Tyr Gly Asp Gly Ala Leu Glu Glu Ala Arg
      145            150            155            160
Arg Leu Arg Glu Gly Asn Trp Ala Ser Val Arg Thr Val Leu Thr Gly
      165            170            175
Ala Val Ala Leu Gly Ala Leu Val Thr Val Gly Ala Phe Phe Ala Ser
      180            185            190
Lys

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 583 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..583

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATG GCG ACC CCA GCC TCG GCC CCA GAC ACA CGG GCT CTG GTG GCA GAC	48
Met Ala Thr Pro Ala Ser Ala Pro Asp Thr Arg Ala Leu Val Ala Asp	
1 5 10 15	
TTT GTA GGT TAT AAG CTG AGG CAG AAG GGT TAT GTC TGT GGA GCT GGC	96
Phe Val Gly Tyr Lys Leu Arg Gln Lys Gly Tyr Val Cys Gly Ala Gly	
20 25 30	
CCC GGG GAG GGC CCA GCA GCT GAC CCG CTG CAC CAA GCC ATG CGG GCA	144
Pro Gly Glu Gly Pro Ala Ala Asp Pro Leu His Gln Ala Met Arg Ala	
35 40 45	
GCT GGA GAT GAG TTC GAG ACC CGC TTC CGG CGC ACC TTC TCT GAT CTG	192
Ala Gly Asp Glu Phe Glu Thr Arg Phe Arg Arg Thr Phe Ser Asp Leu	
50 55 60	
GCG GCT CAG CTG CAT GTG ACC CCA GGC TCA GCC CAG CAA CGC TTC ACC	240
Ala Ala Gln Leu His Val Thr Pro Gly Ser Ala Gln Gln Arg Phe Thr	
65 70 75 80	
CAG GTC TCC GAC GAA CTT TTT CAA GGG GGC CCC AAC TGG GGC CGC CTT	288
Gln Val Ser Asp Glu Leu Phe Gln Gly Gly Pro Asn Trp Gly Arg Leu	
85 90 95	
GTA GCC TTC TTT CTC TTT GGG GCT GCA CTG TGT GCT GAG AGT GTC AAC	336
Val Ala Phe Phe Leu Phe Gly Ala Ala Leu Cys Ala Glu Ser Val Asn	
100 105 110	
AAG GAG ATG GAA CCA CTG GTG GGA CAA GTG CAG GAG TGG ATG GTG GCC	384
Lys Glu Met Glu Pro Leu Val Gly Gln Val Gln Glu Trp Met Val Ala	
115 120 125	
TAC CTG GAG ACG CGG CTG GTC GAC TGG ATC CAC AGC AGT GGG GGC TGG	432
Tyr Leu Glu Thr Arg Leu Val Asp Trp Ile His Ser Ser Gly Gly Trp	
130 135 140	
GCG GAG TTC ACA GCT CTA TAC GGG GAC GGG GCC CTG GAG GAG GCG CGG	480
Ala Glu Phe Thr Ala Leu Tyr Gly Asp Gly Ala Leu Glu Glu Ala Arg	
145 150 155 160	
CGT CTG CGG GAG GGG AAC TGG GCA TCA GTG AGG ACA GTG CTG ACG GGG	528
Arg Leu Arg Glu Gly Asn Trp Ala Ser Val Arg Thr Val Leu Thr Gly	
165 170 175	
GCC GTG GCA CTG GGG GCC CTG GTA ACT GTA GGG GCC TTT TTT GCT AGC	576
Ala Val Ala Leu Gly Ala Leu Val Thr Val Gly Ala Phe Phe Ala Ser	
180 185 190	
AAG TGA A	583
Lys *	

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 194 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Ala Thr Pro Ala Ser Ala Pro Asp Thr Arg Ala Leu Val Ala Asp
 1 5 10 15
 Phe Val Gly Tyr Lys Leu Arg Gln Lys Gly Tyr Val Cys Gly Ala Gly
 20 25 30
 Pro Gly Glu Gly Pro Ala Ala Asp Pro Leu His Gln Ala Met Arg Ala
 35 40 45
 Ala Gly Asp Glu Phe Glu Thr Arg Phe Arg Arg Thr Phe Ser Asp Leu
 50 55 60
 Ala Ala Gln Leu His Val Thr Pro Gly Ser Ala Gln Gln Arg Phe Thr
 65 70 75 80
 Gln Val Ser Asp Glu Leu Phe Gln Gly Gly Pro Asn Trp Gly Arg Leu
 85 90 95
 Val Ala Phe Phe Leu Phe Gly Ala Ala Leu Cys Ala Glu Ser Val Asn
 100 105 110
 Lys Glu Met Glu Pro Leu Val Gly Gln Val Gln Glu Trp Met Val Ala
 115 120 125
 Tyr Leu Glu Thr Arg Leu Val Asp Trp Ile His Ser Ser Gly Gly Trp
 130 135 140
 Ala Glu Phe Thr Ala Leu Tyr Gly Asp Gly Ala Leu Glu Glu Ala Arg
 145 150 155 160
 Arg Leu Arg Glu Gly Asn Trp Ala Ser Val Arg Thr Val Leu Thr Gly
 165 170 175
 Ala Val Ala Leu Gly Ala Leu Val Thr Val Gly Ala Phe Phe Ala Ser
 180 185 190
 Lys *

[illegible][illegible][illegible][illegible][illegible][illegible][illegible][illegible][illegible]

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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 193 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Pro Thr Pro Ala Ser Thr Pro Asp Thr Arg Ala Leu Val Ala Asp
 1 5 10 15
 Phe Val Gly Tyr Arg Leu Arg Gln Lys Gly Tyr Val Cys Gly Ala Gly
 20 25 30
 Pro Gly Glu Gly Pro Ala Ala Asp Pro Leu His Gln Ala Met Arg Ala
 35 40 45
 Ala Gly Asp Glu Phe Glu Thr Arg Phe Arg Arg Thr Phe Ser Asp Leu
 50 55 60
 Ala Ala Gln Leu His Val Thr Pro Gly Ser Ala Gln Gln Arg Phe Thr
 65 70 75 80
 Gln Val Ser Asp Glu Leu Phe Gln Gly Gly Pro Asn Trp Gly Arg Leu
 85 90 95
 Val Ala Phe Phe Val Phe Gly Ala Ala Leu Cys Ala Glu Ser Val Asn
 100 105 110
 Lys Glu Met Glu Pro Leu Val Gly Gln Val Gln Asp Trp Ile Val Ala
 115 120 125
 Tyr Leu Glu Thr Arg Leu Ala Asp Trp Ile His Ser Ser Gly Gly Trp
 130 135 140
 Ala Asp Phe Thr Ala Leu Tyr Gly Asp Gly Ala Leu Glu Asp Ala Arg
 145 150 155 160
 Arg Leu Arg Glu Gly Asn Trp Ala * Val Ser Thr Val Val Thr Gly
 165 170 175
 Ala Val Ala Leu Gly Ala Leu Val Thr Val Gly Ala Phe Phe Ala Ser
 180 185 190
 Lys

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CLAIMS

1. A modified animal or avian species exhibiting reduced levels of a Bcl-w protein and/or protein associated with Bcl-w or a derivative or homologue thereof, wherein said animal or avian species has an incapacity or a reduced capacity to induce or facilitate spermatogenesis.
2. A modified animal or avian species according to claim 1 wherein the Bcl-w protein comprises an amino acid sequence set forth in SEQ ID NO: 2 or SEQ ID NO: 4 or is an amino acid sequence having at least about 47% similarity thereto.
3. A modified animal or avian species according to claim 2 wherein the Bcl-2 is encoded by a nucleotide sequence substantially as set forth in SEQ ID NO: 1 or SEQ ID NO: 3 or is a nucleotide sequence having at least about 47% similarity thereto or is a nucleotide sequence capable of hybridizing to SEQ ID NO: 1 or SEQ ID NO: 3 under low stringency conditions of 42°C.
4. A modified animal or avian species according to claim 1 wherein a protein associated with Bcl-2 is encoded by a gene which is approximately 9.2 kb downstream of *bcl-w* exon 3.
5. A modified animal or avian species according to claim 4 wherein the gene downstream of *bcl-w* is homologous to the *Drosophila rox* gene.
6. A modified animal or avian species according to any one of claims 1 to 5 wherein the modified animal or avian species comprises an introduced antagonist of Bcl-w activity.
7. A modified animal or avian species according to claim 6 wherein the antagonist is an antibody or a Bcl-w binding portion thereof.
8. A modified animal or avian species according to claim 6 wherein the antagonist is a molecule comprising a Bcl-2 Homology (BH) 3 motif.

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9. A modified animal or avian species according to any one of claims 1 to 5 wherein the modified animal comprises a deletion in the *bcl-w* gene.
10. A modified animal or avian species according to any one of claims 1 to 5 wherein the modified animal comprises an introduced genetic molecule capable of inhibiting or reducing exposure of the *bcl-w* gene.
11. A modified animal or avian species according to claim 10 wherein the genetic molecule is an antisense molecule capable of hybridizing to all or part of a mRNA transcription of *bcl-w*.
12. A genetically modified animal comprising a mutation in one or more alleles of a gene comprising a sequence of nucleotides substantially as set forth in SEQ ID NO: 1 or SEQ ID NO: 3 or a nucleotide sequence having at least about 47% similarity thereto and/or a sequence which is capable of hybridizing to SEQ ID NO: 1 or SEQ ID NO: 3 under low stringency conditions at 42°C.
13. A genetically modified animal according to claim 12 comprising a mutation in both alleles of the gene.
14. A method of producing a genetically modified animal substantially incapable of producing Bcl-w, said method comprising introducing a genetic sequence into embryonic stem (ES) cells, which genetic sequence targets the *bcl-w* gene or a transcript thereof or a gene associated with *bcl-w* and introducing said ES cells into blastocysts to produce a chimeric animal.
15. A method according to claim 14 wherein the genetically modified animal is a mouse.
16. A method according to claim 14 or 15 wherein the introduced genetic sequence is an antisense molecule, encoding an antisense molecule or permits excision of the *bcl-w* gene or a region within the *bcl-w* gene.

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17. A method according to claim 16 wherein the introduced genetic sequence encodes the Cre recombinase.

18. A modified animal comprising a mutation in a gene corresponding to *bcl-w* or a derivative or homologue thereof or in a gene associated with *bcl-w* wherein an adult male of said animal exhibits the following characteristics:

- (i) is substantially infertile;
- (ii) possesses disorganised seminiferous tubules;
- (iii) exhibits heterogenous degeneration of germ cell types; and
- (iv) possesses no other major abnormalities as determined by histological examination.

19. A modified animal according to claim 18 wherein the *bcl-w* mutation is on chromosome 14q11.

20. A modified animal or avian species exhibiting reduced levels of a Bcl-w protein having an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:4 or a Bcl-w protein encoded by a nucleotide sequence substantially set forth in SEQ ID NO:1 or SEQ ID NO:3 or a nucleotide sequence capable of hybridising to SEQ ID NO:1 or 3 or 5 or 7 under low stringency conditions at 42 °C wherein said animal or avian species has an incapacity or a reduced capacity to induce or facilitate spermatogenesis.

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ABSTRACT

The present invention relates generally to a method of treatment and to an animal model for the identification of molecules and genetic sequences useful in method of treatment including inducing or reducing fertility of male animals. More particularly, the present invention contemplates a method for the treatment of infertility or a method of reducing fertility and even more particularly a method for modulating spermatogenesis in an animal or avian species. There is also provided an animal model comprising a mutation in at least one allele of *bcl-w* or in a gene associated with *bcl-w*. Such animals fail to undergo productive spermatogenesis and can be used to screen for therapeutic molecules including genetic sequences capable of inducing, enhancing or otherwise facilitating spermatogenesis in said animals as well as a model for molecules and genetic sequences which can induce infertility.

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FIG 1A

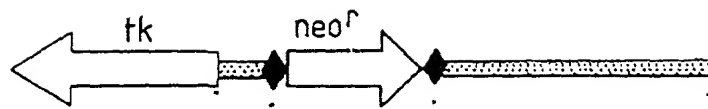


FIG 1B

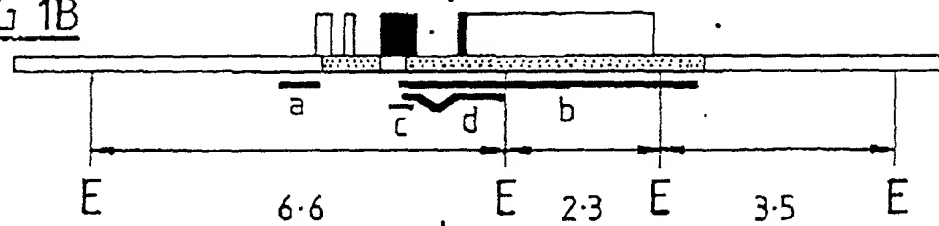


FIG 1C

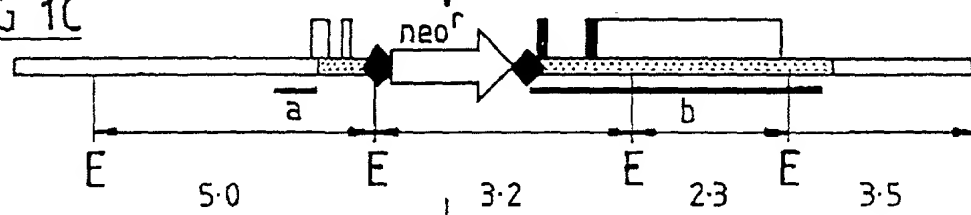
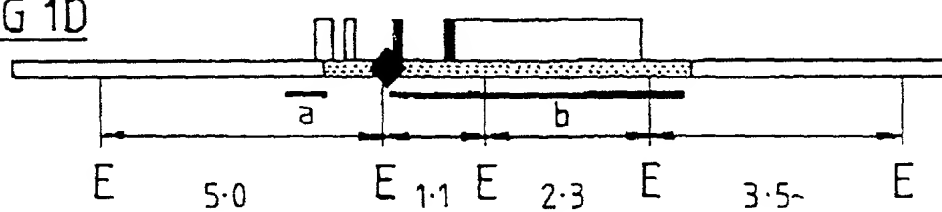


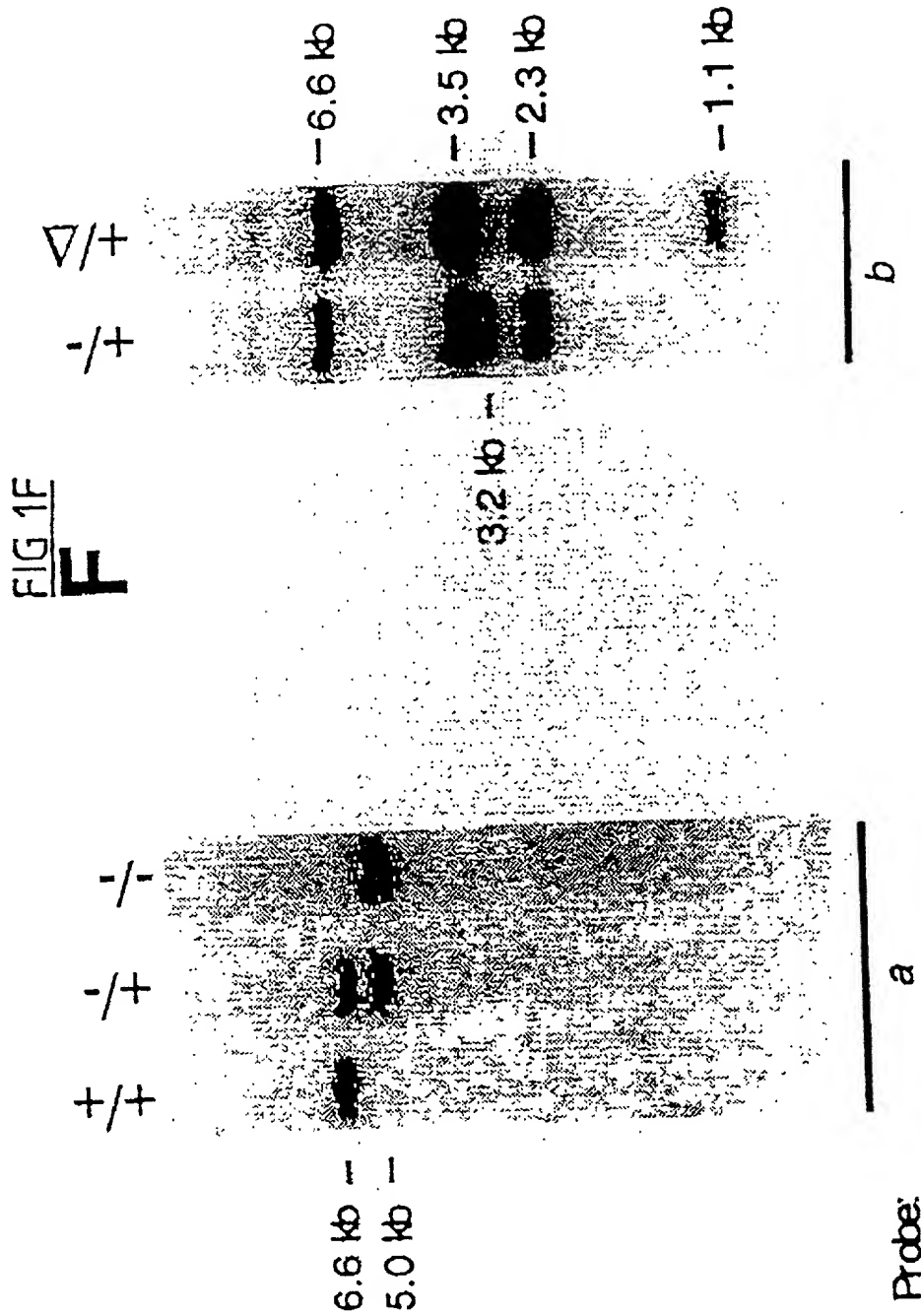
FIG 1D



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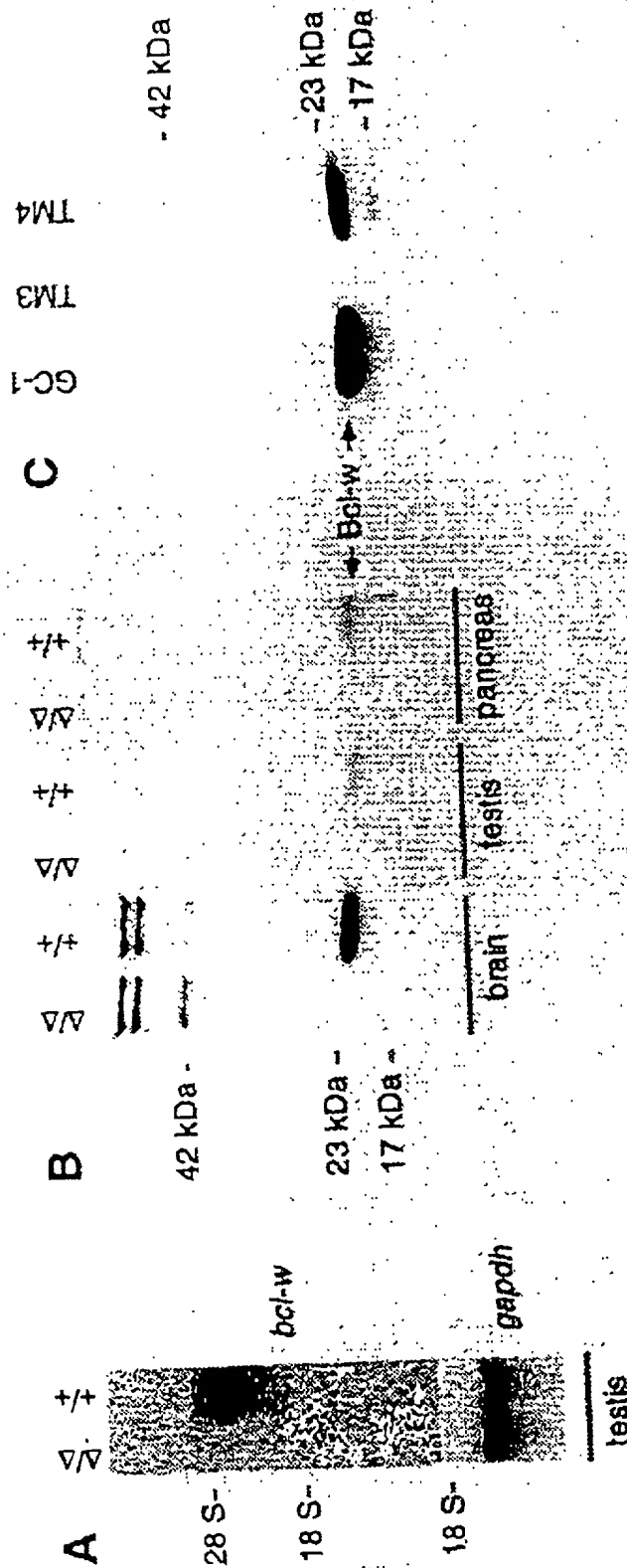
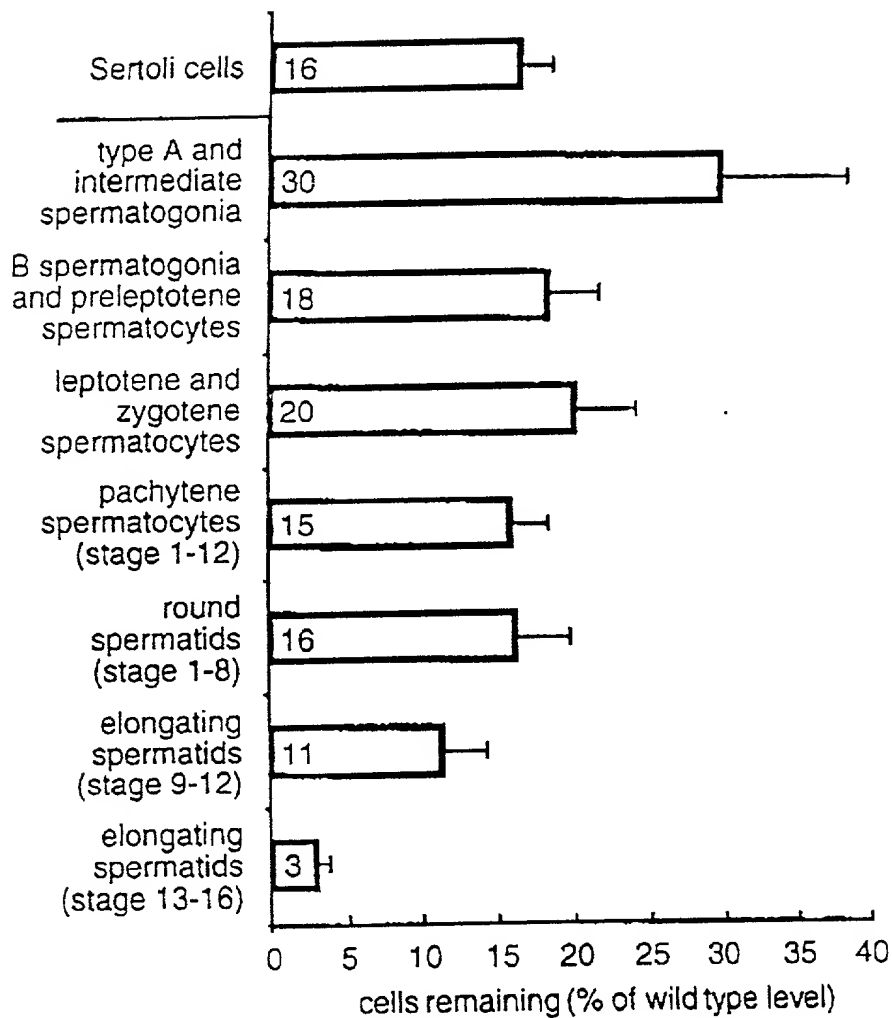


FIG 2

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FIG 3

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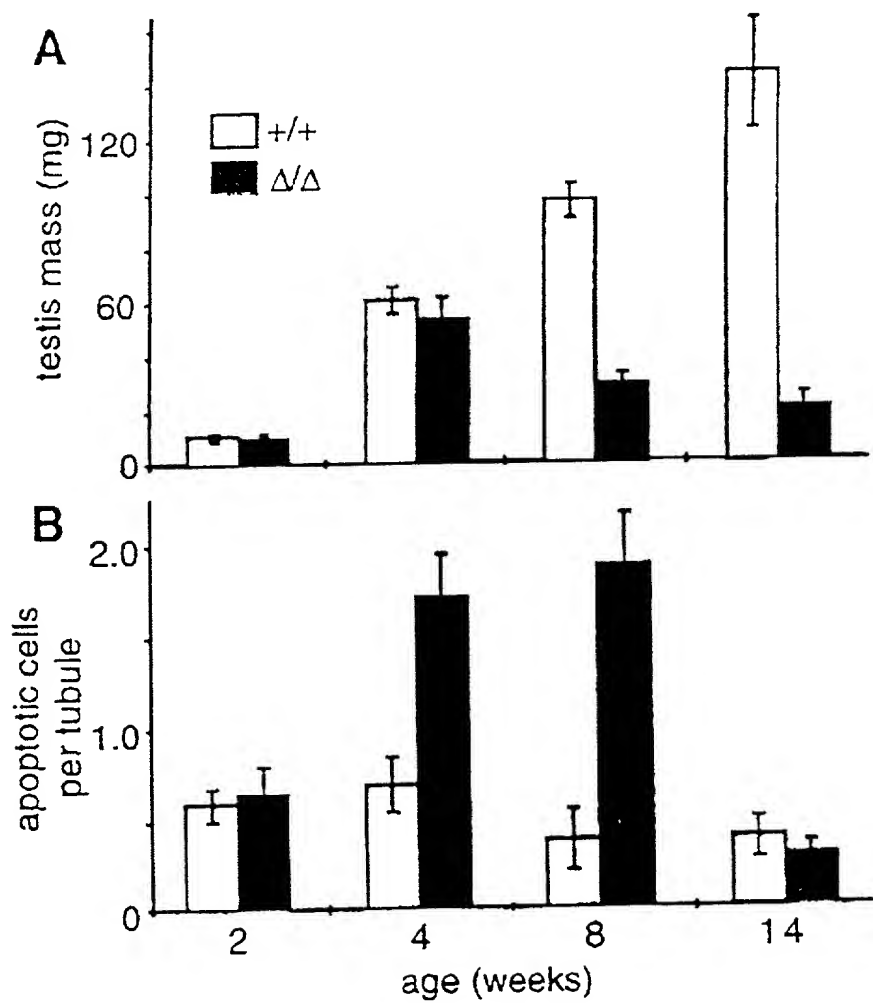


FIG 4

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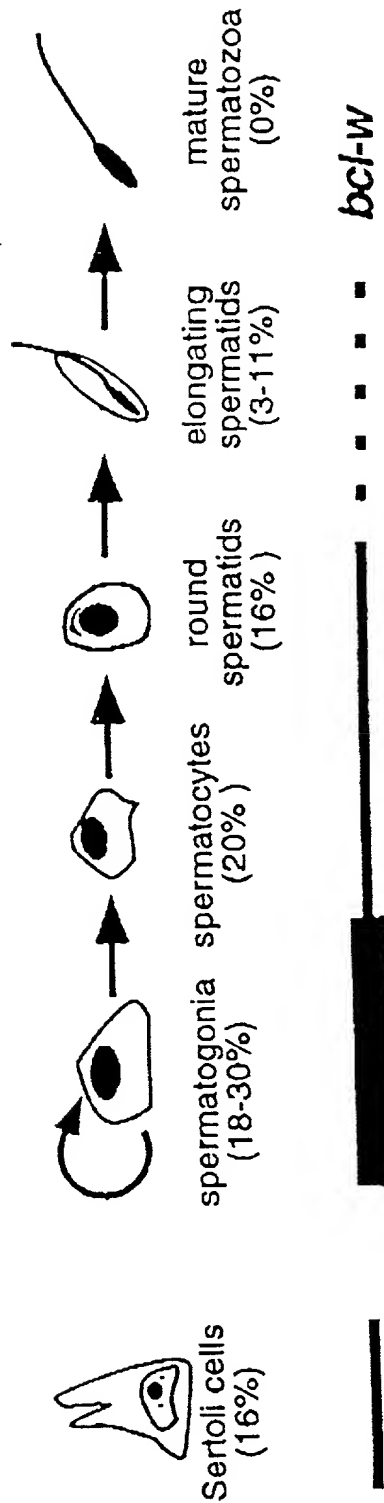
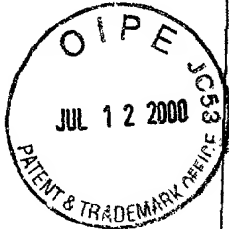


FIG 5



COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

includes the name of the PCT International Application

As a below named inventor I hereby declare that

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

"A METHOD OF TREATMENT AND AN ANIMAL MODEL USEFUL FOR SAME"

the specification of which (check only one item below):

☐ is attached hereto☐ was filed as United States application

Serial No. _____

on _____

and was amended

on _____ (if applicable).

☒ was filed as PCT international applicationNumber PCT/AU98/0076416 September, 1998

on _____

and was amended under PCT Article 19

on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a)

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT, add the PCT #)	APPLICATION NUMBER	DATE OF FILING (day month year)	PRIORITY CLAIMED UNDER 35 U.S.C. 119
AUSTRALIA	PO 9228	16 September, 1997	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

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61 3 9345 2616

ARC-CGF MELB.

004/009

61 3 9345 2616

Combined Declaration For Patent Application and Power of Attorney (Continued)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120

U.S. APPLICATIONS		STATUS (Check one)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED
PCT/AU98/00764	16 September, 1998			
PCT APPLICATIONS DESIGNATING THE U.S.				
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED (if any)		

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Stephen D. Murphy, Reg. No. 22,002; Leopold Presser, Reg. No. 19,827; William C. Roch, Reg. No. 24,972; Kenneth L. King, Reg. No. 24,223; Frank S. DiGiglio, Reg. No. 31,348; Paul J. Esatto, Jr., Reg. No. 30,749; John S. Semany, Reg. No. 28,757; Mark J. Cohen, Reg. No. 32,211; Richard L. Catania, Reg. No. 32,608 and Donald T. Black, Reg. No. 27,999.

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(516) 742-4343

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	CITY	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY
		PRINT	Chris	
		North Melbourne	Victoria, Australia	New Zealand
		46 Erskin Street	North Melbourne, Victoria	3051, Australia

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
x Suzanne Cory	x Jerry Adams	x Chris Print
DATE	DATE	DATE
x	x 14 June, 2000	x 15 June 2000

[] Signature for fourth and subsequent joint inventors.
Number of pages added _____

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Combined Declaration For Patent Application and Power of Attorney (Continued)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

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U.S. APPLICATIONS		STATUS (Check one)		
U.S. APPLICATION NUMBER	FILING DATE	PATENTED	PENDING	ABANDONED
PCT/AU98/00764	16 September, 1998			
PCT APPLICATIONS DESIGNATING THE U.S.				
PCT APPLICATION NO.	FILING DATE	U.S. SERIAL NUMBER ASSIGNED (if any)		

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. Stephen D. Murphy, Reg. No. 22,002; Leopold Presser, Reg. No. 19,827; William C. Roch, Reg. No. 24,972; Kenneth L. King, Reg. No. 24,221; Frank S. DiGiglio, Reg. No. 31,346; Paul J. Esatto, Jr., Reg. No. 30,749; John S. Senany, Reg. No. 28,757; Mark J. Cohen, Reg. No. 32,211; Richard L. Catania, Reg. No. 32,608 and Donald T. Black, Reg. No. 27,999.

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(516) 742-4343

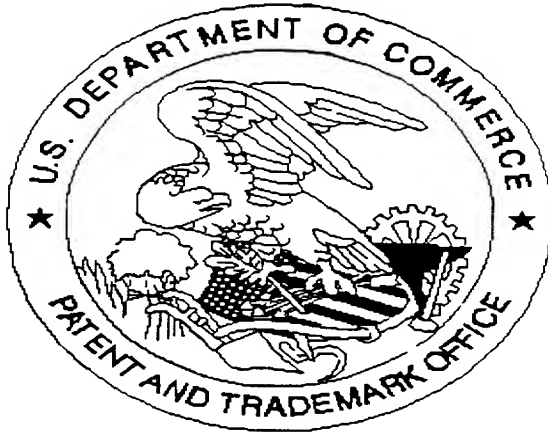
201	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	CITY			
	RESIDENCE & CITIZENSHIP	North Melbourne	Victoria, Australia	Australia
202	POST OFFICE ADDRESS	57 Brougham Street	North Melbourne, Victoria	3051, Australia
	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	CITY	North Melbourne	Victoria, Australia	Australia
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	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
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	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	CITY	North Melbourne	Victoria, Australia	New Zealand

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
<i>Suzanne Cory</i>	<i>Jerry Adams</i>	<i>Chris Print</i>
DATE	DATE	DATE
14 June 2000	14 June, 2000	

() Signature for fourth and subsequent joint inventors.
 Number of pages added _____.

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